Sensitivity of European *Plasmopara viticola* populations to cymoxanil

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Abstract: Between 1995 and 1997, 278 grape downy mildew (*Plasmopara viticola*) populations originating from European vineyards were characterised for their sensitivity to cymoxanil in a leaf-disc assay. The sensitivity profile revealed a wide distribution, with minimum inhibitory concentrations (MIC) ranging from 10 to more than 800 mg litre^{-1}. EC_{50} values ranged from 1 to more than 800 mg litre^{-1} with an average of 125 mg litre^{-1}. The sensitivity distribution was stable between 1995 and 1997. Surprisingly, populations from Portugal appeared significantly more sensitive than those from France or Italy, which could not be linked to differential cymoxanil usage in these countries. *P viticola* populations collected outside Europe and never exposed to cymoxanil appeared significantly more sensitive than exposed European populations, with an average EC_{50} value of 10 mg litre^{-1}. The level of sensitivity of European *P viticola* populations was relatively unaffected by the number of cymoxanil applications made during a season or by the number of years of cymoxanil use. No link was found between the level of sensitivity in the leaf-disc assay and the level of performance of the cymoxanil mixtures used in the fields where the populations originated. Specific field trials conducted in Italy and Portugal have shown that the performance of cymoxanil-based mixtures remained good even on populations of the grape downy mildew fungus characterised as less sensitive in the leaf-disc assay. While there are no baseline sensitivity data for pre-commercialisation *P viticola* populations, the results of our study suggest that a shift in sensitivity (12.5-fold) may have occurred in some areas since introduction of cymoxanil on grapes nearly 20 years ago. Because cymoxanil is never used alone, it is difficult to determine whether or not practical resistance is occurring in European vineyards.

Keywords: cymoxanil; *Plasmopara viticola*; resistance; sensitivity shift; leaf-disc assay

1 INTRODUCTION

The fungicide cymoxanil is the only representative of the cyanoacetamide group. It is used for the control of several oomycete fungi on a variety of crops and is particularly effective on the grape downy mildew fungus (*Plasmopara viticola* (Berk & Curt) Berlese & de Toni).[1] Cymoxanil exhibits strong curative activity in grape downy mildew but relatively short residual properties due to rapid metabolism in the plant tissue.[1,2] For this reason cymoxanil has been used almost exclusively in mixture with contact and/or systemic fungicides. Synergy between cymoxanil and various other active ingredients has been reported.[3–5] Although the biochemical mode of action of cymoxanil is not totally understood, reported studies have suggested that several biochemical processes are disrupted, including the synthesis of nucleic acid and that of several aminoacids.[6] Since the development of resistance to phenylamides,[7] cymoxanil has become a key component on grape downy mildew control strategies. Since its first introduction on the European market nearly 20 years ago, cymoxanil mixtures have been used extensively, usually following a fixed spray schedule. In some areas, downy mildew control strategies recommended by extension services follow a predictive model. In areas such as northern Italy, cymoxanil-based products are sometimes applied only after an infective event, ie relying on the curative properties of cymoxanil.[8] Although the field performance of cymoxanil-based fungicides has remained good in most of Europe, reports have been made of reduced disease control in northern Italy which has been attributed to the development of resistance in *P viticola* to this active ingredient.[8] Unfortunately, no baseline sensitivity data are available to validate this hypothesis. This study was initiated in 1995 to investigate the level of sensitivity of European *P viticola* populations to cymoxanil using the method developed by Genet *et al.*[9] The data obtained with *P viticola* populations never previously exposed to cymoxanil are presented and discussed. The results from specific field trials are also reported.

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2 MATERIALS AND METHODS

2.1 Leaf-disc assay

The sensitivity test was performed on grape leaf discs treated with cymoxanil and inoculated 24 h later with three droplets of a suspension of *P viticola* sporangia. Tested doses of cymoxanil were 3, 10, 30, 60, 100, 300, 600 and 800 mg litre^{-1}. A standard isolate was always used as a reference in each test for validation. The minimum inhibitory concentration (MIC) was recorded and an EC_{50} value (dose needed to reduce growth by 50%) was calculated for each population. Details of the method are described elsewhere.[9]

2.2 Sampling of European populations

Between 30 and 50 grape leaves with symptoms of downy mildew were collected randomly from each of the locations. Samples were collected in the major grape-growing areas of Europe where disease occurred. Between 1995 and 1997 a total of 278 samples were collected in six European countries and characterised for their sensitivity to cymoxanil in the leaf disc assay. Vineyards were sampled regardless of the fungicides used and their level of performance.

When available, information was collected with regard to fungicide use in the vineyard sampled, including the number of cymoxanil applications already made at sampling time and the number of years of use of cymoxanil-based products. An estimate of the level of performance of the fungicides was also provided. The vineyards monitored fell into two categories: (a) sites where disease control was adequate and (b) sites where the overall performance of the products was unexpectedly poor.

Two vineyards in France, one of which had never previously been treated with cymoxanil, were sampled in 1995 and again in 1996 and 1997. One vineyard in Portugal was sampled in 1996 and again in 1997.

2.3 Non-exposed populations from outside Europe

Infected grape leaves were also collected in countries where cymoxanil had never been used, such as Australia, Japan, New Zealand and the USA. A total of 23 grape downy mildew populations were successfully evaluated from these regions.

2.4 Study in Riocaud, France

A specific study was carried out in 1996 to evaluate the effect of repeated applications of cymoxanil mixtures on the sensitivity distribution of individual phenotypes within a *P viticola* population. The field plot (8 ha) located in Riocaud, France, was divided into three parts: area A was left untreated to serve as a season-long inoculum source. Area B was treated exclusively with cymoxanil mixtures and area C was treated with products containing no cymoxanil. The other active ingredients used in combination with cymoxanil in area B were mancozeb, folpet, oxadixyl and copper. The active ingredients used in area C were dimethomorph, mancozeb, maneb and copper. The treated areas were sprayed on the same day with the same application equipment. Samples were collected in area A (untreated) on 10 June. Areas B and C were sampled on 4 September. Five fungicide applications were made at 10–12-day intervals between the two sampling times. Thirty monosporangial isolates were obtained from each of the three samples by means of three successive single sporangium multiplications[10] and analysed in the leaf disc assay.

2.5 Field trials

Field trials were conducted in Conegliano (Italy) and Braga (Portugal) to evaluate the efficacy of the mixture cymoxanil + mancozeb (40 + 400 g kg^{-1} WP) for the control of grape downy mildew. The mixture was applied at label rate ie 2.5 kg ha^{-1} in Italy and 3 kg ha^{-1} in Portugal. The efficacy of the mixture was compared to that provided by mancozeb (800 g kg^{-1} WP) applied at its dose in the mixture. The trials were performed according to EPPO guidelines[11] under a natural infection.

3 RESULTS

3.1 European populations

The sensitivity distribution to cymoxanil of grape downy mildew populations originating from Europe is relatively broad (Fig 1). The MIC values varied from 10 to more than 800 mg litre^{-1} (the water solubility of cymoxanil precluded the use of concentrations higher than 1000 mg litre^{-1}). A small percentage of the populations (13%) were inhibited at 100 mg litre^{-1} or less. Sixty-four percent of the populations tested had an MIC between 300 and 800 mg litre^{-1} and a significant number (23%) were still growing at 800 mg litre^{-1}. The EC_{50} values ranged from 1 to more than 800 mg litre^{-1}, with a mean value of 125 mg litre^{-1}. The sensitivity profile of European populations of *P viticola* was stable between 1995 and 1997 (Fig 2).

Whereas the sensitivity profiles of the *P viticola* populations from France and Italy were quite similar populations from Portugal appeared significantly more sensitive to cymoxanil (Fig 3). Portuguese populations had generally a sharper dose-response curve with 58% of them having an EC_{50} value lower than 10 mg litre^{-1}. The mean EC_{50} value for these populations was 31 mg litre^{-1}. Interestingly, MIC values of 800 mg litre^{-1} or greater were also found among Portuguese populations (data not shown). Table 1 compares the usage of cymoxanil based products in the vineyards of France, Italy and Portugal which were sampled between 1995 and 1997. The number of applications of cymoxanil products was significantly higher in Italy compared to France or Portugal (Table 1A). The total number of downy mildew fungicides applied during the season was also

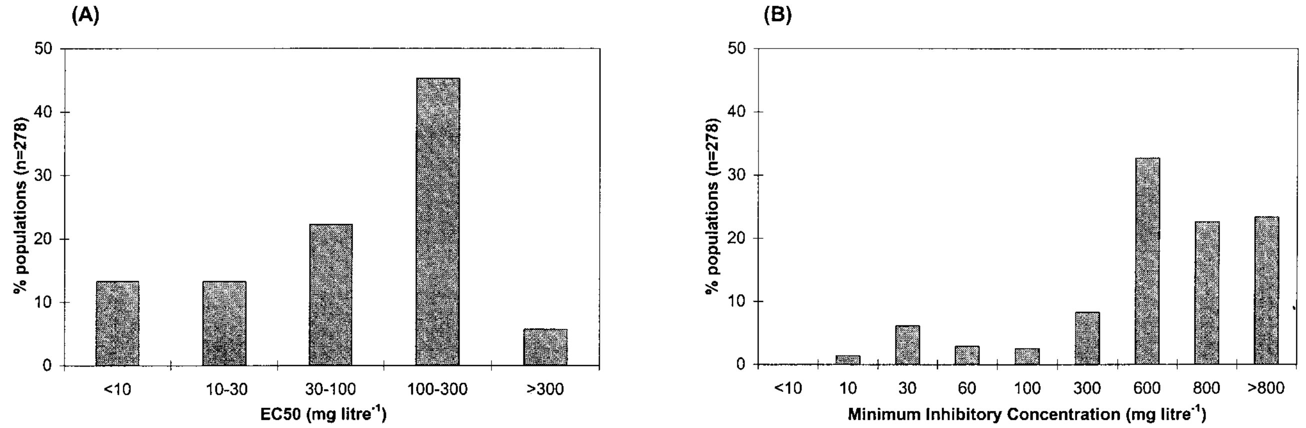

Figure 1. Sensitivity distribution to cymoxanil of European *Plasmopara viticola* populations sampled between 1995 and 1997. (A) EC_{50} values (B) MIC values.

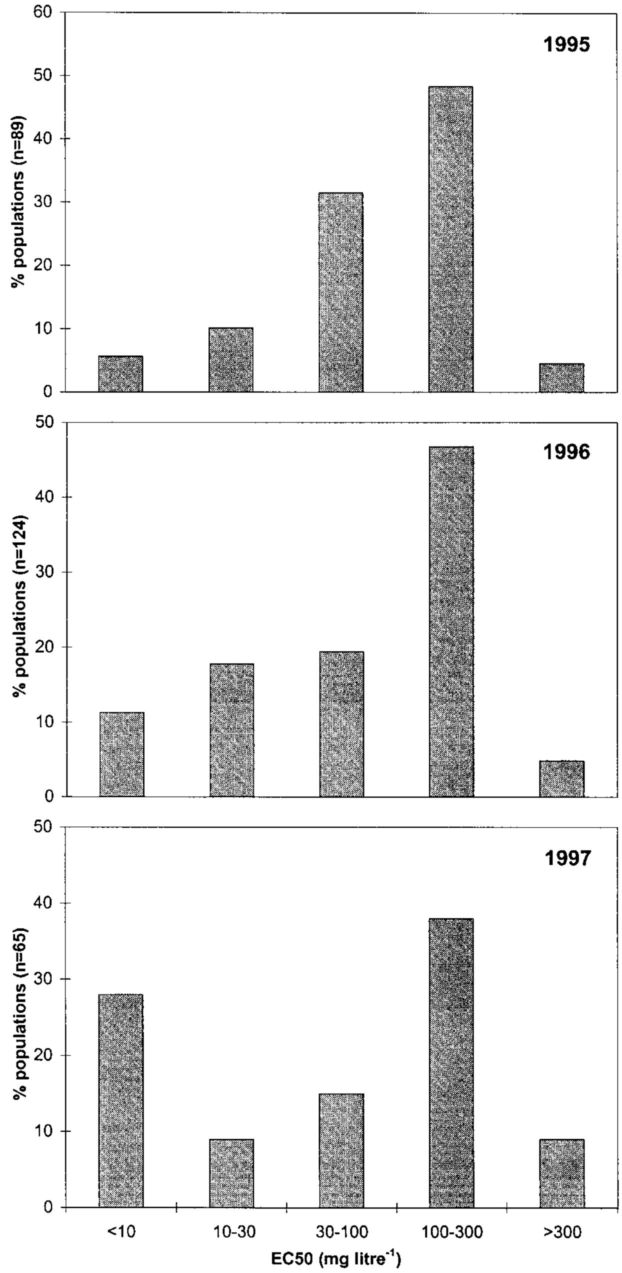

Figure 2. Distribution of EC_{50} values of cymoxanil for European *Plasmopara viticola* populations sampled in 1995, 1996 and 1997.

highest in Italy, possibly reflecting a generally higher disease pressure in this country (data not shown). The number of years with cymoxanil use was highest in France compared to Italy and Portugal (Table 1B).

The *P viticola* populations collected in the various regions of France exhibited similar sensitivity distributions to cymoxanil, with mean EC_{50} values of 128 to 165 mg litre^{-1} (Table 2).

The sensitivity distribution of *P viticola* populations isolated from fields treated with cymoxanil was the same whether the overall fungicide performance evaluated at sampling time was considered adequate (Fig. 4A) or unexpectedly low (Fig 4B). Performance failures could not be linked to a reduced sensitivity of the populations in those fields.

Table 1. Use of cymoxanil-based mixtures for the control of *Plasmopara viticola* in sites monitored for sensitivity between 1995 and 1997 in France, Italy, and Portugal. The values represent the percentage of sites. (A) Number of cymoxanil sprays made before sampling. (B) Number of years with cymoxanil use.

A

Country	*No of sites*	*Number of cymoxanil applications*			
		0	*1–4*	*5–9*	*≥10*
France	155	28	44	20	8
Italy	37	14	16	43	27
Portugal	36	25	42	30	3

B

Country	*No of sites*	*Number of years with cymoxanil use*			
		0	*1–4*	*5–9*	*≥10*
France	79	8	13	21	58
Italy	25	0	32	52	16
Portugal	28	11	28	43	18

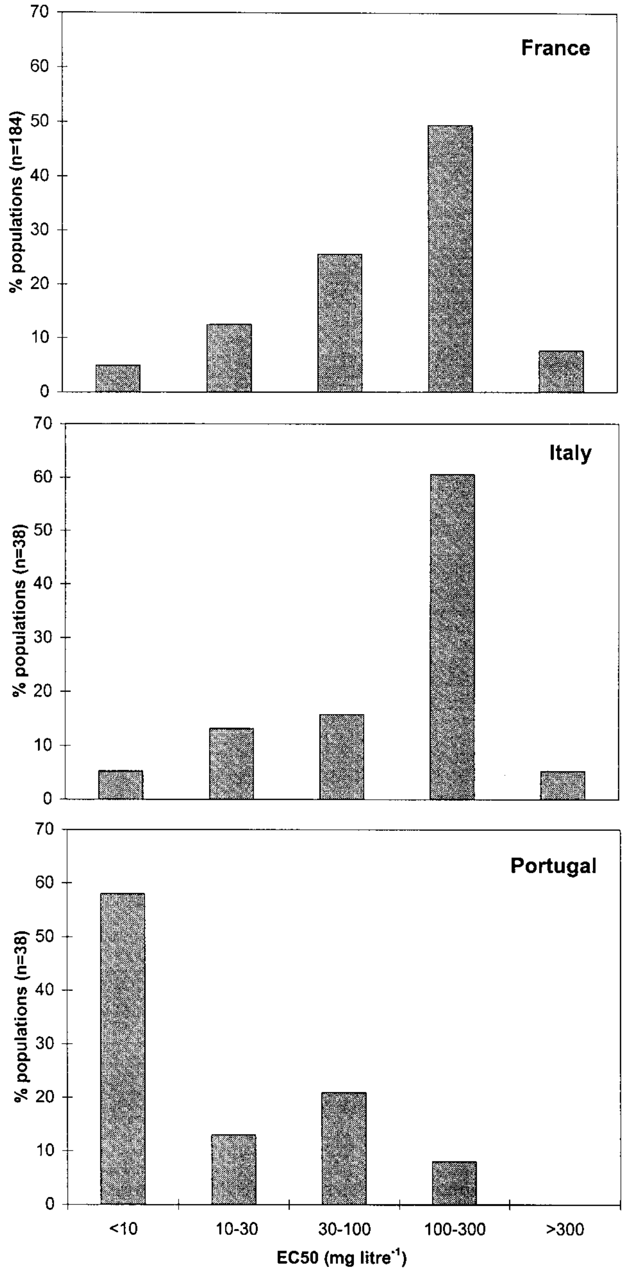

Figure 3. Distribution of EC_{50} values of cymoxanil for European *Plasmopara viticola* populations collected in 1995, 1996 and 1997 in France, Italy and Portugal.

Similarly, there was no correlation ($r^2 = 0.083$) between isolate sensitivity and years of cymoxanil exposure (Fig 5).

Table 2. Sensitivity to cymoxanil of *Plasmopara viticola* populations collected in various regions of France between 1995 and 1997

Region	*No of isolates*	*EC_{50} (mg litre^{-1})*	
		Mean	*Range*
East	31	132	5–761
West	24	165	10–424
South east	40	153	1–409
South west	76	128	1–933

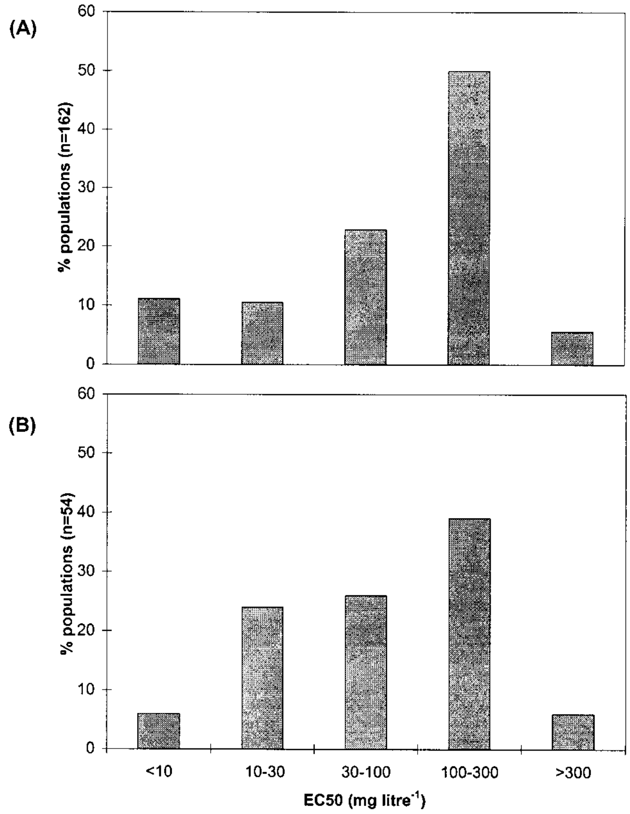

Figure 4. Distribution of EC_{50} values of cymoxanil for European *Plasmopara viticola* populations collected in fields treated with cymoxanil mixtures. (A) Sites where disease control was adequate (B) Sites where the efficacy of the fungicide sprays was questioned.

The sensitivity of *P viticola* populations to cymoxanil was relatively unaffected by the number of cymoxanil applications that had been made during the season before samples were collected (Table 3). The mean EC_{50} value for populations collected at the end of the season in vineyards treated 10 times or more with products containing cymoxanil was 136 mg litre^{-1}, compared to 90 mg litre^{-1} for populations isolated in vineyards treated with other products or untreated.

When the same vineyard was sampled two or three years consecutively, the level of sensitivity of the grape downy populations isolated from that field was found to be unstable (Table 4). For example, in a

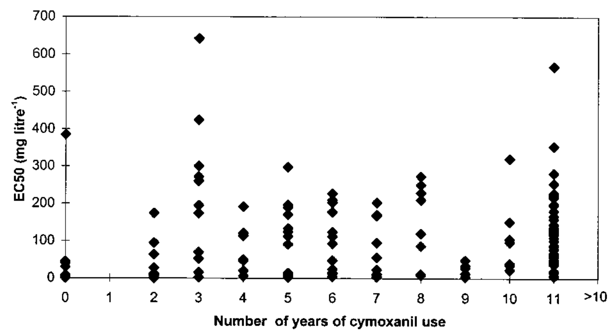

Figure 5. Sensitivity to cymoxanil of *Plasmopara viticola* populations relative to the number of years of cymoxanil use. Samples collected between 1995 and 1997.

Table 3. Sensitivity to cymoxanil of *Plasmopara viticola* populations relative to the number of cymoxanil applications during the season. Samples collected between 1995 and 1997 in European countries

No. of cymoxanil applications[a]	*No of sites*	*EC_{50} (mg litre^{-1})*	
		Mean	*Range*
0	60	90	1–471
1–4	91	123	1–761
5–9	63	132	5–354
$\geq$10	23	136	2–642

[a] Before sample collection.

field never treated with downy mildew fungicides (Pessac, France), the population was found to be very sensitive in 1995 (MIC = 30 mg litre^{-1}) then less sensitive in 1996 (MIC = 600 mg litre^{-1}) and again very sensitive in 1997 (MIC = 30 mg litre^{-1}). These findings suggest that mixing of populations occurs over relatively larger distances.

3.2 Non-exposed populations from outside Europe

The frequency distribution of EC_{50} values among 23 populations that had never been previously exposed to cymoxanil (Fig 6A) was significantly narrower than that of exposed European populations (Fig 1). The mean EC_{50} value for non-European populations was 10 mg litre^{-1}, compared to 125 mg litre^{-1} in European populations. The frequency distribution of MIC values among non-exposed populations was remarkably different from that of exposed populations from Europe (Fig 6B). The distribution of MIC values was apparently bimodal, but the relatively small sample size does not allow making definitive conclusions. Fifty-three percent of the non-exposed populations had a MIC value between 10 and 100 mg litre^{-1}, compared to 13% for exposed populations. Also, all non-exposed populations were inhibited at the highest rate in the assay (800 mg litre^{-1}).

3.3 Study in Riocaud, France

A monosporangial isolate of *P viticola* is thought to represent one phenotype.[10] Analysis of 30 monosporangial isolates obtained from the untreated area A at the beginning of the season revealed a relatively narrow distribution of sensitivities (phenotypes) within the population (Fig 7A). Five applications of cymoxanil-based products during the season in area B had a limited influence on this distribution (Fig 7B). Interestingly, new phenotypes with low MICs (3 and 10 mg litre^{-1}) were found in September in the cymoxanil-treated area B (data not shown). These very sensitive phenotypes were also found in the area treated exclusively with fungicides containing no cymoxanil (area C). The occurrence of these phenotypes in September and their absence at the beginning of the season may be explained by a migration from neighbouring fields or sampling variation. The population isolated from area B contained fewer of

Table 4. Sensitivity to cymoxanil of *Plasmopara viticola* populations collected in the same vineyard in 1995, 1996 and 1997

Location	*Year of sampling*	*Sampling date*	*MIC (mg litre^{-1})*	*EC_{50} (mg litre^{-1})*
Jonzac	1995	29 Aug	800	151
(France)	1996	16 Sep	600	105
	1997	28 July	>800	471
Pessac	1995	20 Aug	30	5
(France)	1996	27 Jul	600	84
	1997	25 Jun	30	5
Torres Vedras	1996	29 Jul	300	64
(Portugal)	1997	29 Jul	800	15

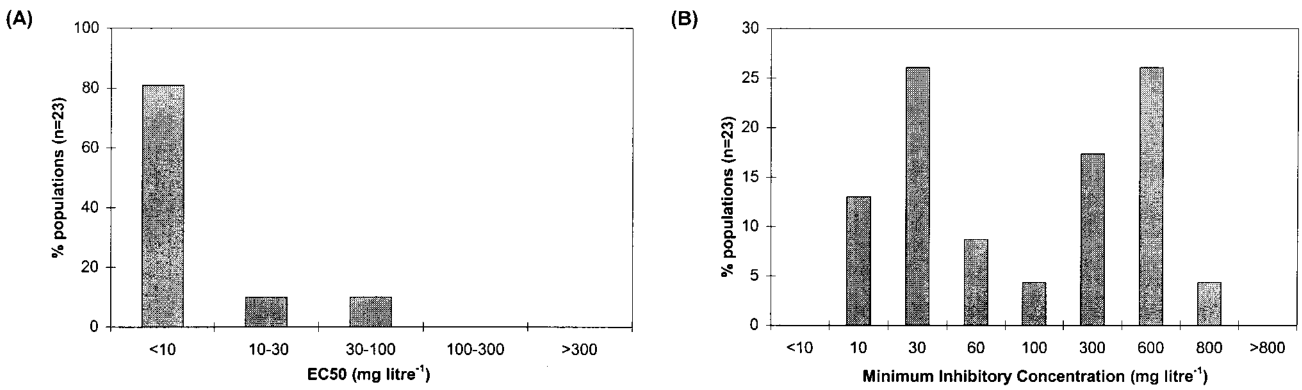

Figure 6. Sensitivity distribution to cymoxanil of *Plasmopara viticola* populations never previously exposed to cymoxanil. (A) EC_{50} values. (B) MIC values. Samples collected between 1995 and 1997 in Australia, Japan, New Zealand and the USA.

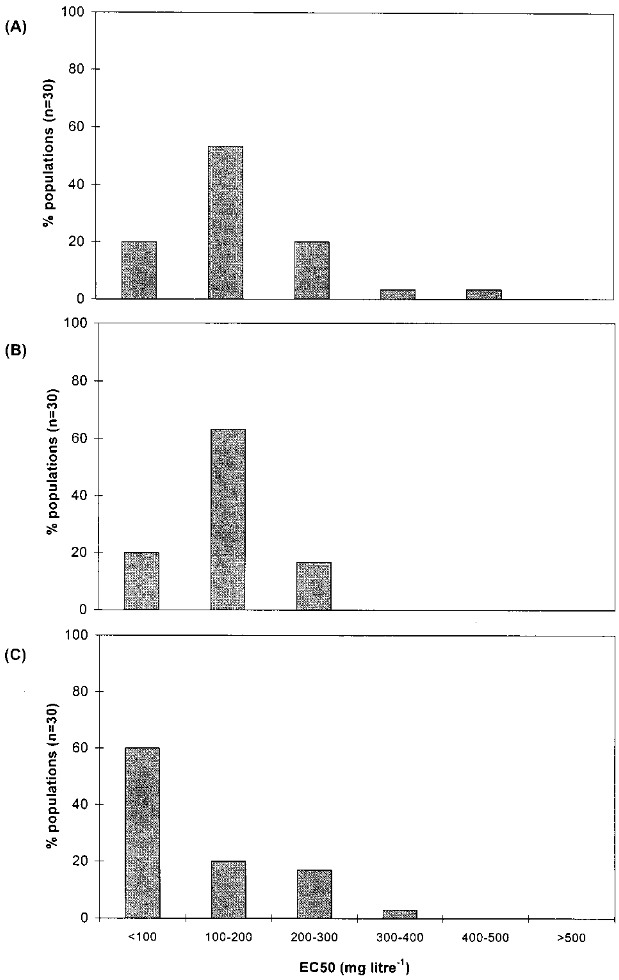

Figure 7. Sensitivity distribution of 30 monosporangial isolates of *Plasmopara viticola* from a vineyard in Riocaud, France. (A) Samples collected in June from an untreated area. (B) Samples collected in September from an area treated five times with cymoxanil. (C) Samples collected in September from an area treated five times with fungicides other than cymoxanil.

the more sensitive phenotypes (<100 mg litre^{-1}) compared to area C (Fig 7B and 7C), probably as a result of the selection pressure. The same classes of sensitivities were found in these two areas, suggesting that selection pressure had only limited effects on the population in the cymoxanil-treated field.

3.4 Field trials

The grape downy mildew epidemic was much more severe in Conegliano (north-east Italy) than in Braga (Portugal). As a result, fungicide treatments were more frequent in Italy (13 applications) than in Portugal (8 applications). The disease pressure resulted in almost complete destruction of untreated bunches in the Italian trial compared to only 21% destruction in Portugal (Table 5). The *P viticola* population found in Braga was more sensitive to cymoxanil in the leaf-disc assay (MIC = 60 mg litre^{-1}) compared to the population isolated in Conegliano (MIC = 800 mg litre^{-1}). All fungicide treatments provided significant disease control, including mancozeb applied at half its normal rate. In both locations the performance of the mixture cymoxanil + mancozeb was significantly superior to that of mancozeb alone ($P > 0.05$) even under the extremely high disease pressure of Conegliano (Table 5).

4 DISCUSSION AND CONCLUSION

Cymoxanil has generally been considered at low risk for developing resistance. Its biochemical mode of action, although incompletely explained, involves several biosynthetic processes.[6,12] Attempts to induce laboratory mutants of *Phytophthora spp.* resistant to cymoxanil have failed (Leroux P, 1981, pers comm). Studies on *Phytophthora infestans* (Mont) de Bay have led to the conclusion that there has been no significant decrease in sensitivity to cymoxanil after more than 16 years of exposure.[13] Finally, cymoxanil has been used exclusively in mixtures, which are considered to be a key element in anti-resistance strategies.[14]

Three years (1995 to 1997) of intensive monitoring in European populations of *P viticola* have indicated a wide variation in sensitivity to cymoxanil, which is in agreement with the results from other studies[8] (Gisi U, 1996, pers comm). The sensitivity profile revealed a unimodal but somewhat asymmetric distribution with a factor of nearly 1000 between the

Table 5. Field performance of cymoxanil applied in mixture for the control of *Plasmopara viticola* in Braga (Portugal and Conegliano (Italy). Disease level estimated visually by percentage of infection

Location	*No of applications*	*Active ingredient used*	*Rate (g AI ha^{-1}*	*Infection* (%)[b]	
				Bunches	*Leaves*
Braga[a]	8	cymoxanil + mancozeb	120 + 1400	0.98 c	—
		mancozeb	1400	5.8 b	—
		untreated	–	21 a	—
Conegliano[b]		cymoxanil + mancozeb	100 + 1000	16.5 c	0.3 c
		mancozeb	1000	38.4 b	0.5 b
		untreated	–	98 a	42.5 a

[a] Disease level evaluated on 23 Aug 1996.
[b] Disease level evaluated on 6 Aug, 1996.
[c] Values followed by the same letter within a column at each location are not significantly different based on LSD at $P > 0.05$.

most and the least sensitive population (Fig 1). In *P infestans*, variation factors of 1000 have also been found for cymoxanil.[15]

It is very difficult to reach conclusions on the significance of this variation, as no true baseline is available from Europe. In an attempt to generate a baseline, non-exposed populations of downy mildew have been collected outside Europe. These populations appeared to be generally more sensitive to cymoxanil than contemporary exposed populations from Europe. Assuming that these non-exposed populations represent the sensitivity levels that existed within the original populations from Europe, one might conclude that a shift in sensitivity has occurred. However, this possible shift, recorded nearly 20 years after the introduction of cymoxanil on grapes, remains small, with only a 12.5-fold difference in mean EC_{50} values between exposed and unexposed populations. In comparison, a difference in the order 1,000-fold has been reported for metalaxyl between sensitive and resistant phenotype.[7] The magnitude of the shift observed for cymoxanil and the continuous distribution of sensitivities in contemporary populations of *P viticola* suggest that the mechanisms involved are probably under the control of several genes.[16]

More surprising is the geographical distribution of the sensitivity to cymoxanil across Europe. Whereas populations from the various regions of France showed similar sensitivity profiles (Table 2), populations from Portugal appeared significantly more sensitive than those of France or Italy (Fig 3). This could only partially be explained by the history of cymoxanil usage in these countries (Table 1). In Portugal, cymoxanil-based products are generally recommended as preventive sprays, as in France. This is, however, not the case in Italy, where post-infection applications are often advised.[8] Other factors such as climate, grapevine cultivars or genetic differences in *P viticola* might also be involved.

The studies conducted in Riocaud, France have shown that repeated applications of cymoxanil-based products during one season resulted in little change in the sensitivity of the exposed population at the end compared to the beginning of the season (Fig 7). This, and the situation encountered in France and Italy, indicates that the grape downy mildew populations seem to have stabilised. However, a long-term monitoring programme is in place to detect further changes in the overall sensitivity to cymoxanil of *P viticola* populations in Europe.

Earlier studies conducted in the glasshouse have shown that selected *P viticola* populations with low MIC values in the leaf-disc assay were also better controlled in a whole-plant test than were populations with high MIC values, indicating good agreement between the two methods.[9] Both types of assay, however, rely on a single inoculation with the fungus following one single fungicide application, whereas, in the field, multiple applications and infections occur. In addition, these laboratory experiments were conducted with cymoxanil alone, whereas, in practice, the fungicide is used in mixtures, where synergy has been reported.[3–5] Our field trials have shown that cymoxanil mixtures used in a preventive way provided downy mildew control at a level clearly superior to that of the companion fungicide alone, even on *P viticola* populations less sensitive to cymoxanil in the leaf-disc assay (Table 5). These findings differ from those of Gullino *et al*,[18] who reported a reduction in disease control from post-infection sprays of cymoxanil-based fungicides against less sensitive populations of *P viticola* in Italy.

Because cymoxanil is always used in mixtures, it is difficult to determine whether or not practical resistance to this compound is occurring in European vineyards. There is no strong evidence from the data available that the presence in a vineyard of *P viticola* populations with high MIC values will lead to a loss of performance when cymoxanil mixtures are used preventively under practical field conditions. In order to reduce the risk of resistance, cymoxanil-based products should be recommended in pre-infection application schedules.

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